FORM PTO-1390 (REV. 12-2001) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: NEX85/PCT-US

U S APPLICATION NO. (If known, see 37 CFR 1.5): 10/030677

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/20625	28 JULY 2000	29 JULY 1999

TITLE OF INVENTION: NUCLIEC ACID LIGANDS TO CD40LIGAND

APPLICANT(S) FOR DO/EO/US: KORMAN, Alan; GOLD, Larry

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☑ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☑ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☑ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☑ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☑ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☑ Other items or information:
    Abstract; Application Data Sheet; Postcard Receipt; Statement Under 37 C.F.R. §1.821; Paper copy of sequence listing (pages 1-21)

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/030677	PCT/US00/20625	NEX85/PCT-US

21. ☑ The following fees are submitted:	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 710.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	21 - 20 =	1	x **$18.00**	$ 18.00
Independent claims	10 - 3 =	7	x **$84.00**	$ 588.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$
TOTAL OF ABOVE CALCULATIONS			=	$ 1,316.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$
SUBTOTAL			=	$ 1,316.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE			=	$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$
TOTAL FEES ENCLOSED			=	$ 1,316.00
			Amount to be refunded:	$
			charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 22-0277 in the amount of $ 1316.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 22-0277 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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January 11, 2002
SIGNATURE

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39,726
REGISTRATION NUMBER

EXPRESS MAIL LABEL NO - EV 001126299 US
DOCKET NO. NEX 85/PCT

IN THE UNITED STATES RECEIVING OFFICE
(RO/US)

U.S. APPLICATION SERIAL NO.: 10/_____

U.S. FILING DATE: JANUARY 11, 2002

INTERNATIONAL APPLICATION NO.: PCT/US00/20625

INTERNATIONAL FILING DATE: 28 JULY 2000

PRIORITY DATE CLAIMED: 29 JULY 1999

FOR: NUCLEIC ACID LIGANDS TO CD40LIGAND

APPLICANT: GILEAD SCIENCES, INC.

Assistant Commissioner for Patents
Washington, D.C. 20231
Box: PCT

STATEMENT UNDER 37 C.F.R. § 1.821(f)

Dear Sir:

Enclosed is a computer readable form of the SEQUENCE LISTING for the above-identified application submitted under 37 C.F.R. § 1.821(e). A paper copy of the SEQUENCE LISTING has been submitted under 37 C.F.R. § 1.821(c).

I hereby state that the contents of the disk and the paper copy are the same. 37 C.F.R. § 1.821(f).

The paper and disk copies of the SEQUENCE LISTING include no new matter. 37 C.F.R. § 1.821(g).

Respectfully submitted,

Date: January 11, 2002

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NUCLEIC ACID LIGANDS TO CD40LIGAND

Field of the Invention

This invention is directed to a method for the generation of nucleic acid ligands having specific functions against target molecules using a method known as Systematic Evolution of Ligands by EXponential enrichment (SELEX). The invention is directed towards nucleic acid ligands of CD40ligand.

Background of the Invention

CD40ligand (also known as CD154) is a member of the TNF family of molecules. It is a type II membrane protein (N-terminus intracellular and C-terminus extracellular) that is expressed on activated T cells. The human protein is 261 residues long and has a single N-linked carbohydrate moiety. Antibodies to CD154 have been shown to suppress T cell and antibody mediated immune responses in a number of experimental systems. These include inhibition of graft rejection and blocking autoimmune disorders (Durie *et al.* (1993) Science 261:1328). The combined use of anti-CD40ligand antibodies and CD28 blockers (i.e. CTLA-4Ig) has been shown to be effective in blocking graft rejection in both murine and rhesus transplant models (Larsen *et al.* (1996) Nature 381:434; Kirk (1997) Proc. Natl. Acad. Sci. 94:8789). More recently, the use of anti-CD40ligand antibody as a single agent in rhesus kidney allografts has shown that this treatment is remarkably efficacious (Kirk *et al.* (1999) Nature Medicine 5:686.).

CD40ligand is also expressed on activated platelets and this observation has kindled interest in the role of CD40ligand-CD40 interactions in vascular biology (Henn *et al.* (1998) Nature 391:591). CD40 and CD40 ligand expression has also been reported on vascular endothelium and smooth muscle cells (Mach *et al.* (1997) Proc. Natl. Acad. Sci. 94:1931). One report has suggested that inhibition of CD40ligand:CD40 interactions may diminish the development of atherosclerotic lesions (Mach *et al.* (1998) Nature 394:200). Atherosclerosis has been viewed as a disease state in which inflammatory processes of the immune system may play a role. Given the potential therapeutic results of inhibiting the activity of the CD40ligand, it would be desirable to have high affinity and high specificity inhibitors of this molecule.

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential

enrichment, termed the SELEX process, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. The SELEX process is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in U.S. Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned, U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands," and U.S. Patent No. 5,270,163 (see also WO 91/19813), entitled "Methods for Identifying Nucleic Acid Ligands," each of which is specifically incorporated herein by reference in its entirety. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

The SELEX process provides a class of products which are referred to as nucleic acid ligands or aptamers, each having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified nucleic acid ligand is a specific ligand of a given target compound or molecule. The SELEX process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets. The SELEX method applied to the application of high affinity binding involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as

many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that nucleic acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by nucleic acids in biological systems.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, U.S. Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and U.S. Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, U.S. Patent No. 5,763,177 and U.S. Patent No.6,011,577, both entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. U.S. Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified nucleic acid ligands containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands

Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. U.S. Patent No. 5,580,737, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). U.S. Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S. Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chimeric SELEX," and U.S. Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in U.S. Patent No. 6,011,020, entitled "Nucleic Acid Ligand Complexes." Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

It is an object of the present invention to provide methods that can be used to identify nucleic acid ligands that bind with high specificity and affinity to CD40ligand.

It is a further object of the present invention to obtain nucleic acid ligands to CD40ligand that inhibit the activity of CD40ligand when bound.

Summary of the Invention

The present invention includes methods of identifying and producing nucleic acid ligands to human CD40ligand and the nucleic acid ligands so identified and produced. The method uses the SELEX process for the Systematic Evolution of Ligands by

EXponential enrichment. In particular, RNA sequences are provided that are capable of binding specifically to a human CD40. Also included are oligonucleotides containing nucleotide derivatives modified at the 2' position of the pyrimidines. Specifically included in the invention are the RNA ligand sequences shown in Tables 2 and 3 (SEQ ID NOS:1-31). The nucleic acid ligands of the invention can inhibit the interaction of CD40ligand with its receptor CD40. High affinity nucleic acid ligands to CD40ligand have many potential uses in the treatment and diagnosis of diseases of the immune system.

Brief Description of the Drawings

Figure 1 shows the structure of the recombinant forms of human and mouse CD40ligand proteins used as SELEX targets in the instant invention.

Figure 2 shows inhibition of binding of a murineCD8-human CD40ligand fusion protein to the surface of Raji cells (CD40-positive) by nucleic acid ligands to CD40ligand (p40).

Figure 3 illustrates the binding of biotinylated RNA pools and aptamers to hCD40L-expressing Lkt cells.

Detailed Description of the Invention

The central method utilized herein for identifying nucleic acid ligands to CD40ligand is called the SELEX process, an acronym for Systematic Evolution of Ligands by Exponential enrichment and involves: (a) contacting the candidate mixture of nucleic acids with CD40ligand, or expressed domains or peptides corresponding to CD40ligand; (b) partitioning between members of said candidate mixture on the basis of affinity to CD40ligand; and (c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to CD40ligand.

Definitions

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided.

As used herein, "**nucleic acid ligand**" is a non-naturally occurring nucleic acid having a desirable action on a target. Nucleic acid ligands are often referred to as "**aptamers**." A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In a preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand does not have the known physiological function of being bound by the target molecule. In the present invention, the target is CD40ligand, or regions thereof. Nucleic acid ligands include nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

As used herein, "**candidate mixture**" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

As used herein, "**nucleic acid**" means either DNA, RNA, single-stranded or double-stranded, and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications

include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"**SELEX**" methodology involves the combination of selection of nucleic acid ligands that interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to CD40ligand.

The SELEX methodology is described in the SELEX Patent Applications.

"**SELEX target**" or "**target**" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this application, the SELEX target is CD40ligand. In particular, the SELEX targets in this application include purified CD40ligand, and fragments thereof, and short peptides or expressed protein domains comprising CD40ligand. Also included as targets are fusion proteins comprising portions of CD40ligand and other proteins, such as murine CD8.

As used herein, "**solid support**" is defined as any surface to which molecules may be attached through either covalent or non-covalent bonds. This includes, but is not limited to, membranes, microtiter plates, magnetic beads, charged paper, nylon, Langmuir-Bodgett films, functionalized glass, germanium, silicon, PTFE, polystyrene, gallium arsenide, gold, and silver. Any other material known in the art that is capable of having functional groups such as amino, carboxyl, thiol or hydroxyl incorporated on its surface, is also contemplated. This includes surfaces with any topology, including, but not limited to, spherical surfaces and grooved surfaces.

As used herein "**CD40ligand**" refers to the ligand for CD40. "CD40ligand" also refers to CD154. This includes purified ligand, fragments of ligand, chemically synthesized fragments of the ligand, derivatives or mutated versions of the ligand, and fusion proteins comprising the ligand and another protein. "CD40ligand" as used herein also includes the ligand of the CD40 receptor isolated from a species other than humans.

Note, that throughout this application various citations are provided. Each citation is specifically incorporated herein in its entirety by reference.

Preparation of nucleic acid ligands to CD40ligand

In the preferred embodiment, the nucleic acid ligands of the present invention are derived from the SELEX methodology. The SELEX process is described in U.S. Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment, " now abandoned, U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands," and U.S. Patent No. 5,270,163 (see also WO 91/19813), entitled "Methods for Identifying Nucleic Acid Ligands." These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products that are nucleic acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired target compound or molecule. Target molecules are preferably proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX methodology can also be used to target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series of steps.

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below; (b) to mimic a sequence known to bind to the target; or (c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the

probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, U.S. Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and U.S. Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands." now abandoned, U.S. Patent No. 5,763,177 and U.S. Patent No. 6,001,577, both entitled

"Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," all describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. U.S. Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. U.S. Patent No. 5,496,938, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. U.S. Patent No. 5,705,337, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. U.S. Patent No. 5,637,459, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). U.S. Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S.

Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," and U.S. Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

In U.S. Patent No. 5,496,938, methods are described for obtaining improved nucleic acid ligands after the SELEX process has been performed. This patent, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," is specifically incorporated herein by reference.

One potential problem encountered in the diagnostic use of nucleic acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes, such as endonucleases and exonucleases, before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the *in vivo* stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. See, e.g., U.S. Patent Application Serial No. 08/117,991, filed September 8, 1993, now abandoned and U.S. Patent No. 5,660,985, both entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," and U.S. Patent Application Serial No. 09/362,578, filed July 28, 1999, entitled "Transcription-free SELEX," each of which is specifically incorporated herein by reference in its entirety. Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases, isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. In preferred embodiments of the instant invention, the nucleic acid ligands are

RNA molecules that are 2'-fluoro (2'-F) modified on the sugar moiety of pyrimidine residues.

The modifications can be pre- or post-SELEX process modifications. Pre-SELEX process modifications yield nucleic acid ligands with both specificity for their SELEX target and improved *in vivo* stability. Post-SELEX process modifications made to 2'-OH nucleic acid ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligand.

Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX process (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

The nucleic acid ligands of the invention are prepared through the SELEX methodology that is outlined above and thoroughly enabled in the SELEX applications incorporated herein by reference in their entirety. The SELEX process can be performed using purified CD40ligand, or fragments thereof as a target. Alternatively, full-length CD40ligand, or discrete domains of CD40ligand, can be produced in a suitable expression system. Alternatively, the SELEX process can be performed using as a target a synthetic peptide that includes sequences found in CD40ligand. Determination of the precise number of amino acids needed for the optimal nucleic acid ligand is routine experimentation for skilled artisans.

In some embodiments, the nucleic acid ligands become covalently attached to their targets upon irradiation of the nucleic acid ligand with light having a selected wavelength. Methods for obtaining such nucleic acid ligands are detailed in U.S. Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, U.S. Patent No. 5,763,177 and U.S. Patent No. 6,001,577, both entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," each of which is specifically incorporated herein by reference in its entirety.

In preferred embodiments, the SELEX process is carried out using fragments of CD40ligand that are bound to magnetic beads through hydrophobic interactions. A candidate mixture of single stranded RNA molecules is then contacted with the magnetic beads in the wells of a microtiter plate. After incubation for a predetermined time at a

selected temperature, the beads are held to the sides of the wells of the plate by a magnetic field, and the wells of the plate are washed to remove unbound candidate nucleic acid ligands. The nucleic acid ligands that bind to the CD40ligand are then released into solution in the wells, then reverse transcribed by reverse transcriptase and amplified using the Polymerase Chain Reaction (PCR). The amplified candidate mixture is then used to begin the next round of the SELEX process.

The nucleic acid ligands isolated by the method of the present invention can then be tested for binding to CD40ligand protein and for inhibition of the interaction between CD40ligand and CD40. This can be done, for example, by assaying the nucleic acid ligand for inhibition of the proliferation of B cells that is normally induced by CD40ligand and IL-4. Alternatively, the nucleic acid ligands, and CD40ligand or a CD40ligand-fusion protein, can added to cells positive for the CD40 protein, and the inhibition of the interaction can be seen by immunofluorescence using an antibody directed towards an irrelevant portion of the protein, such as murineCD8 in a murineCD8-humanCD40ligand fusion protein. Inhibition of the interaction between soluble CD40ligand and CD40 expressed at the cell surface results in diminished fluorescent staining at the cell surface, or none at all.

Diagnostic and therapeutic applications of CD40ligand nucleic acid ligands

The nucleic acid ligands provided by the instant invention are useful in a number of medical applications. For example, they can be used to treat or diagnose any disease in which T cell activity, antibody-mediated immune responses, or activated platelets play a role in pathogenesis. In some embodiments, they can used in patients who have received an organ transplant or a graft in order to block organ or graft rejection. In other embodiments, the nucleic acid ligands are used to reduce the development of vascular diseases, such as the development of atherosclerotic lesions. In still further embodiments, the nucleic acid ligands are used to treat autoimmune disorders. In order to use nucleic acid ligands as therapeutic agents, it may be necessary to use modified nucleotides and ribonucleotides in order to impart increased stability upon the nucleic acid ligand in biological fluids. Such modifications are described above in the SELEX patent applications.

The nucleic acid ligands of the instant invention can also be used to image blood clots formed by platelet aggregation. Patients susceptible to thrombosis --because of major trauma or surgery-- can be injected with radiolabeled nucleic acid ligands to CD40ligand, and then radioimaging can reveal sites in the body where large aggregations of platelets, and hence thrombi, are present. If a thrombosis is detected at a critical site in the body, then anticoagulant and thrombolytic treatment can be given locally. The advantage of using such a nucleic acid ligand imaging agent is that the anticoagulant and thrombolytic treatments --which can cause harm if administered prophylactically by allowing internal bleeding to continue without efficient clotting-- can be given only to those individuals who definitely have a dangerous thrombosis. Moreover, these treatments can be specifically injected at the site where the thrombosis has been detected by the nucleic acid ligands of the instant invention, instead of injecting higher concentrations into the bloodstream in the hope that some active agent will be carried to all potential sites of thrombosis.

Therapeutic compositions of the nucleic acid ligands may be administered parenterally by injection, although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment, it is envisioned that the carrier and the ligand constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder.

Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing nucleic acid ligands for systemic delivery may be via subcutaneous, intramuscular, intravenous, intranasal or vaginal or rectal suppository.

The following Examples are provided to explain and illustrate the present invention and are not intended to be limiting of the invention.

Examples

Example 1. Generation of CD40ligand for use as target in the SELEX process

CD40ligand was expressed in *E. Coli* as a fusion protein. The fusion protein comprised amino acid 108 to amino acid 261 (the carboxy terminus) of human CD40ligand fused at the NH_2-terminus to a 6-his sequence followed by a thrombin cleavage site. The resulting fusion protein, p20, was purified by binding to a Ni-NTA column. p18 was then generated by treatment of p20 with biotinylated thrombin, followed by removal of the biotinylated thrombin with streptavidin agarose followed by dialysis.

CD40ligand was also expressed in eukaryotic cells --both COS and CHO cells-- as a murineCD8-human (or mouse) CD40ligand fusion protein. The resulting protein, p40/p50 was purified by anti CD8-Sepharose chromatography. A variant of this construct (p40) contains an internal deletion that removes residues 50-108 of the CD40ligand protein; this eliminates two free cysteine residues and results in a protein with a reduced level of intermolecular crosslinking.

In addition, to the proteins described above, human CD40ligand was expressed in transfected L cells using a full length cDNA encoding the mature CD40ligand protein. The structure of the various expressed CD40ligand proteins is shown in Figure 1.

The p18 and p40/p50 proteins were demonstrated to have biological activity. p18 was able to induce B cell proliferation in the presence of IL-4. Anti-CD40ligand antibodies inhibited this proliferation. This shows that the B cell proliferative activity was not due to bacterial lipopolysaccharides; in addition, a control protein (6-his-beta-galactosidase) purified in a similar manner did not have B cell proliferative activity. The p40 /p50 was also shown to stimulate B cell proliferation in the presence of IL-4. In addition, p40/p50 was shown to bind to CD40-positive Raji cells and normal B cells by

use of a secondary reagent directed to the CD8 portion of the molecule. Figure 2 shows data for the human CD40L fusion protein. Data for the murine CD40L fusion protein is not shown.

Example 2. Generating nucleic acid ligands to p18 and p40

The SELEX method was performed using both p18 and p40 proteins for human CD40L and p40 protein for mouse CD40L. Proteins were bound to magnetic beads by hydrophobic absorption. The beads were contacted with a candidate mixture of single stranded 2'-fluoro-pyrimidine (2'-F) RNA, and incubated to allow the candidate nucleic acid ligands to bind to the p18 or p40 protein immobilized on the beads. The beads were then washed to remove unbound candidate nucleic acid ligands, and the beads were partitioned from the unbound nucleic acid ligands by applying a magnetic field. The nucleic acid ligands that bound to p18 or p40 were then eluted from the beads. The eluted nucleic acid ligands were reverse transcribed, and the resulting DNA templates were amplified using the polymerase chain reaction. The amplified DNA templates were then transcribed to yield a candidate mixture of nucleic acid ligands enriched for nucleic acid ligands that bind to p18 or p40. This process was repeated for a total of 6 rounds.

The progress of the different SELEX rounds is shown in Table 1. After six rounds nucleic acid ligand pools were tested in several assays. RNA from round 6 of the human p40 SELEX experiment was used to bind to muCD8-humanCD40 ligand fusion protein; the binding data revealed that affinity for the round 6 pool had improved ~2 logs relative to the round 0 unselected pool. Binding to an irrelevant CD8 fusion protein was negligible (data not shown).

Example 3. Sequences of clones obtained from the SELEX method using p40 and p18 as targets

The sequences of the clones from the p40 selections and the p18 selection are shown in Tables 2-4 (SEQ ID NOS:3-70). The nomenclature in column 1 indicates the species of origin (h = human, m = mouse), protein construct (p18 or p40), the SELEX round (R) from which the nucleic acid ligand was obtained and the clone identification number following the decimal. Hence, hP40R6.3 indicates clone number 3 obtained from

round 6 of the SELEX experiment using human p40 as the target. Note that each aptamer shown in Tables 2-4 has the 40N7 fixed sequence gggaggacgaugcgg (SEQ ID NO:1) at the 5' end, and the 40N7 fixed sequence cagacgacucgcccg (SEQ ID NO:2) at the 3' end. "U" or "C" in any sequence corresponds to 2'-F-U or 2'-F-C, respectively. "A" or "G" corresponds to 2'-OH-A or 2'-OH-G, respectively. An "N" indicates that the nucleotide in that position is ambiguous (i.e., may be either 2'-OH-A or -G, or 2'-F-C or -U). In Table 4, "Y" corresponds to either 2'-F-U or 2'-F-C, "R" corresponds to either 2'-OH-A or 2'-OH-G, "M" corresponds to either 2'-OH-A or 2'-F-C, and "S" corresponds to either 2'-OH-G or 2'-F-C.

Example 4. Inhibition of the binding of the murineCD8-humanCD40ligand fusion protein to CD40-positive cells

RNA from round 6 for the p18 (p18R6 pool) and human p40 SELEX experiments was prepared and tested for its ability to inhibit the binding of the murineCD8-humanCD40ligand fusion to CD40-positive cells (as revealed by binding of fluorescent anti-CD8-PE antibody to the cells). MurineCD8-humanCD40ligand (mCD8-hCD40ligand) at a concentration of 0.25 μg/mL was incubated with 1 x 10 e-5 Raji cells (CD40-positive) and incubated for 1 hour at room temperature followed by three washes and subsequent incubation with anti-mCD8-PE antibody (obtained from Pharmingen). At this concentration of mCD8-huCD40ligand, a mean channel fluorescence (MCF) value of ~8 was obtained (see Figure 2). Inhibition of this interaction was performed as follows: nucleic acid ligands, antibodies, and Ig fusions (humanCD40-human Ig and human CD5-human Ig) were incubated with the indicated concentrations with 0.25 μg/mL of mCD8-hCD40ligand in a 50 μL reaction for ½ hour at 37°C followed by the addition of Raji cells (1×10^5) cells. The incubation was continued at 37°C for 1 hour followed by three washes with PFA (PBS; 10% Fetal Calf Serum; sodium azide 0.01%). The cells were then incubated in PFA containing fluorescently labeled anti-murine CD8 antibody (PE anti-mCD8) for 1 hour and washed three times prior to analysis by Fluorescence Activated Cell Sorting (FACS) using a Coulter Facstar.

Clones from each of the human CD40L SELEX pools were also analyzed. Two round 6 clones, hP40R6.8 (SEQ ID NO:20) and hP18R6.7 (SEQ ID NO:22), were found

to be strong inhibitors of the CD40ligand interaction (see Figure 2). These nucleic acid ligands were unable to inhibit the binding of murine p40 (murineCD8-murine CD40ligand) (data not shown), demonstrating that the nucleic acid ligand does not bind to the CD8 sequence and is not crossreactive with murine CD40ligand. Also shown in Figure 2 are the binding curves for hP18R6.5 (SEQ ID NO:28), the entire round 6 pool for the p18 SELEX experiment, human CD40-human Ig fusion protein (hCD40-hIg), human CD5-human Ig fusion protein (hCD5-hIg), anti-humanCD40ligand antibody (anti-hCD40L), and anti-murineCD8 antibody (PE anti-mCD8).

The inhibitory aptamers (hP40R6.8 and hP18R6.7) represented ~10-15% of the sequences obtained. Binding analysis using the hP40R6.8 aptamer gave an affinity of the aptamer for the human p40 protein of ~200pM; however, the binding curve was biphasic and the plateau RNA binding was poor (~15%).

Example 5. Staining of Ltk cells with biotinylated nuclei acid ligands

Ltk cells were transfected for expression of the human CD40ligand gene; one clone was selected for high level constitutive expression. These cells were incubated with biotinylated nucleic acid ligands at the concentrations indicated in Figure 3. The biotinylated human p40 nucleic acid ligands used were: round 5 nucleic acid ligands (hP40R5 RNA pool-biotin), hP40R6.1 (SEQ ID NO:5), hP40R6.4 (SEQ ID NO:15), hP40R6.8 (SEQ ID NO:20), hP40R6.40 (SEQ ID NO:21) and hP40R6.48 (SEQ ID NO:11). The initial candidate mixture (Random 40N7 RNA) was also biotinylated and assayed. Anti-human CD40ligand antibody (anti-hCD40L-biotin) (obtained from Pharmingen) was used as a control. After incubation for 1/2 hours at 4°C, the cells were washed once and incubated with fluorescently-labeled streptavidin (SA-PE) for 1 hour at 4°C. Cells were washed three times in PFA and analyzed. The results (Figure 3) show that biotinylated aptamers are able to bind to humanCD40ligand transfectants.

Table 1. Selection parameters and results for six rounds of the SELEX method using human or murine p40 protein or human p18 protein.

hCD40L p40 SELEX

Round	Beads (μL)	RNA (pmol)	[RNA] (M)	RNA bound (molecules)	Background (molecules)	S/B	Molecules RNA bound/μl beads
1	50	290	2.9e-6	1.4e9	2.8e8	5	2.8e7
2	50	195	2.0e-6	6.4e8	1.3e8	5	1.3e7
3	25	200	2.0e-6	4.9e10	2.5e8	196	2.0e9
4	5	200	2.0e-6	1.8e10	1.6e7	1125	3.6e9
5	0.5	300	3.0e-6	3.9e8	8.3e7	5	7.8e8
6	0.25	250	2.5e-6	4.0e8	3.5e7	11	1.6e9

hCD40L p18 SELEX

Round	Beads (μl)	RNA (pmol)	[RNA] (M)	RNA bound (molecules)	Background (molecules)	S/B	Molecules RNA bound/μl beads
1	50	290	2.9e-6	1.2e11	2.8e8	429	2.4e9
2	16.7	195	2.0e-6	9.6e10	2.7e7	3556	5.7e9
3	3	200	2.0e-6	1.4e10	1.1e8	127	4.7e9
4	0.6	200	2.0e-6	2.4e9	1.0e9	2.4	4.0e9
5	0.3	150	1.5e-6	5.8e8	5.8e8	17	1.9e9
6	0.1	250	2.5e-6	5.6e8	5.6e8	15	5.6e9

mCD40L p40 SELEX

Round	Beads (μl)	RNA (pmol)	[RNA] (M)	RNA bound (molecules)	Background (molecules)	S/B	Molecules RNA bound/μl beads
1	50	300	3.0e-6	1.2e10	4.3e8	28	2.4e8
2	25	174	1.7e-6	4.7e10	4.9e8	96	1.9e9
3	2.5	250	2.5e-6	2.2e10	2.4e8	92	8.8e9
4	0.25	200	2.0e-6	1.7e9	1.1e8	15	6.8e9
5*	1	100	1.0e-6	1.4e10	2.7e7	519	1.4e10
6	0.25	100	1.0e-6	1.4e10	9.5e7	147	5.6e10

*A fresh batch of mCD40L-coated beads was prepared prior to the fifth round of selection.

Table 2. Aptamer sequences derived from round 6 of SELEX to murineCD8-human CD40ligand fusion protein (p40). Each aptamer has 40N7 fixed sequence (SEQ ID NO:1) at the 5' end and (SEQ ID NO:2) at the 3' end.

Clone name	Selected Sequence Region	SEQ ID NO
hP40R6.3	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCUACCN	3
hP40R6.9	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCUCCCA	4
hP40R6.1	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCUACCA	5
hP40R6.13	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCAACCU	6
hP40R6.17	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCUACCC	7
hP40R6.29	AUUUGCGAGAAAGGAGCCUCUUAAGACCAACCAUCCGCC	8
hP40R6.34	CAAGAAAGGAACGUUCAGUCAACCAUCUGCUACCGCUCCC	9
hP40R6.44	CAAGAAAGGAACGUUCAGUCAACCAUCUGCUACCGCCCCC	10
hP40R6.48	AGAAAGGAAGAACUCUCUCAACCAUCCCACACCAGCCCCC	11
hP40R6.2	CAACUCUCGAGAAAGGAACAUCAAAGUGUCAACCAUCCGU	12
hP40R6.19	CCUAAGAAAGGAAUUUAAACCAAUCAACCAUCUAGAACCC	13
hP40R6.28	GCCUCGAGAAAGAACCAUACAGGGUAAUCAUCCGUUCGCC	14
hP40R6.4	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCACUAGCCC	15
hP40R6.7	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCACUACCCC	16
hP40R6.25	UCAACCAUCCAACUUAAGCUGAGAAAGGAACCACAAGCCC	17
hP40R6.30	UCAACCAUCCAACUCAAGCUGAGAAAGGAACCACUCGCCC	18
hP40R6.16	AUUGAUCAACCAUCCAGCAAGCUGAGAAAGGAACCAACCU	19
hP40R6.8	AUCUACGCACUCGCAAAAGCAUCAAAUGUGUCCGCCGCCU	20
hP40R6.40	UUAGACAANUGNACNAANNGAAUCNANCCANUCCCNNC	21

Table 3. Aptamer sequences derived from round 6 of Selex to human p18 –*E.Coli* produced CD40ligand. Each aptamer has 40N7 fixed sequence (SEQ ID NO:1) at the 5' end and (SEQ ID NO:2) at the 3' end.

Clone name	Selected Sequence Region	SEQ ID NO
hP18R6.7	AAUGUUUAUUCAUAGAACAGGGUCUACUCAUCACAUCCCC	22
hP18R6.22	AAUGUUUUGUUNNGNNAUUACNAANNUUACUGNCUAUNCU	23
hP18R6.14	CCCCAACGACAGAACAACUCCACAACUGUGCAGUCCCCCG	24
hP18R6.17	CCCCAACGACAGAACAACUCAUAACUGUGCAGUCCCCCG	25
hP18R6.11	CAUCAGCAUAUAACGGACAACGAGCAUACAUUUCACGCGC	26
hP18R6.3	CCACAUCACUCUCUCACUCCCAUUGAAUACUUACCCUCCC	27
hP18R6.5	AUUCCCCUCUCCUGUGUAACCUUCCUUCUCUUCACCUCCU	28
hP18R6.2	AACCCGGGCAGUCCAAUCUUUCAGUCCCACAUCUGCUCCC	29
hP18R6.1	CAAACCUCCACAACCUGCGCAAGCUUCCCAUCNUUCUGCC	30
hP18R6.21	ACGUCUCGGUUGAGCCCUUACAAUCAUUUCUGUAUGCCCU	31
hP18R6.9	ACGAAGACCCGAUGGCCCAGAGAAAUCUCCCACUCUGCCC	32
hP18R6.15	AAGCCCUAAGAGAAUAGCCCUUGACGCCUACUCCCCUGCC	33

Table 4. Aptamer sequences derived from round 6 of SELEX to murineCD8-murine CD40ligand fusion protein (p40). Each aptamer has 40N7 fixed sequence (SEQ ID NO:1) at the 5' end and (SEQ ID NO:2) at the 3' end.

Clone name	Selected Sequence Region	SEQ ID NO
mP40R6.F6	ACAAGUGAUCUGAGGAAGGAAGACAAGACUCUCAACCACC	34
mP40R6.H6	ACAAGAGAUCUGUGGAAGGAAGACAAGACUCUCAACCACC	35
mP40R6.E9	AACUCUCGAGAAAGGAACAUCAAAGUGUCAACCAUCCGU	36
mP40R6.A5	CAAGAAAGGAACGUUCAGUCAACCAUCUGCUACCGCCCU	37
mP40R6.F2	CAACUCUCGAGAAAGGAACAUCAAAGUGUCAACCAUCCGU	38
mP40R6.E4	GAACUCUCGAGAAAGGAACAUCAAAGUGUCAACCAUCCGU	39
mP40R6.H9	AGAAAGGAGAGAAAAUCCAACCAUCCCGAACACGCUCCCU	40
mP40R6.F5	AGGAAGGAACGAUAGAAGUCAACCACCCCACAGUUGCCCU	41
mP40R6.A6	CAACUCUCGAGAAAGGAACAUCAAAGCGUCAACCAUCCGU	42
mP40R6.A10	CAACUCUCGAGAAAGGAAUCACAUAGAUCAACCACCCGGU	43
mP40R6.E11	CAACUCUCGAGAAAGGAACAUCAAAGUGUCAACCAUCUGU	44
mP40R6.C10	CACUGCUCGAGGAAGGAAUCACAUAGAUCAACCACCCGU	45
mP40R6.C6	CAAGAAAGGAACUCUAAUGGUCAACCAUCUGCGCGCACCU	46
mP40R6.D11	CAAGAAAGGAACGUUCAGUCAACCAUCUGCUAUCGCCCU	47
mP40R6.H8	CAAGAAAGGAACGUUCAGUCAACCAUCUGCUACCGUCCU	48
mP40R6.D1	CAAGAAAGGAAUAGCAUGACCGGCUUUCAACCAUCUGGU	49
mP40R6.C12	CCAAGAAAGGAAUAGCAUGACCGGCUUUCAACCAUCUGGU	50
mP40R6.E7	CCAAGAAAGGAAUAGCAUGACAGGCUUUCAACCAUCUGGU	51
mP40R6.D7	CUCGAGAAAGGAACAAAGGUCAACCAUCCGAGCCCUACCU	52
mP40R6.A8	GCUCAGCAAGAACAAAGGCUGGGGUAAAUUUCCGACCCGU	53
mP40R6.A12	GCUCAGUAAGAACAAAGGCUGGGGUAAAUUUCCGACCCGU	54
mP40R6.A3	GCUCAGCAAGAACAAAGGCUGGGGUAAAUUUCCAACCCGU	55
mP40R6.G5	GGACACCGGAUACCCCCCGGCGUGUAAAUUUCAAGCCCCC	56
mP40R6.B3	GGACACCGGAUACCCCCCGGMGUGUAAAUUMCAAGCCCCC	57
mP40R6.B12	GCUCCCAGAGAUUGCUGGGGGGUAAAUUUCCAACCCGU	58
mP40R6.G6	GCUCCCAGAGAUUGCUGGGGGGUAAAUUUCCGACCCGU	59
mP40R6.A2	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCGCUAGCCC	60
mP40R6.G10	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCACUAGCCC	61
mP40R6.H1	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCACUCGCCC	62
mP40R6.B6	NCANNCANCCANCNNAAGNNGAGAAAGGAACCACNACCCC	63
mP40R6.B10	UCAACCAUCCAACUCAAGUUGAGAAAGGAACCGCUACCCC	64
mP40R6.A4	UCAACCAUCCAACUCAAGUAGAGAAAGGAACCGCUAGCCU	65
mP40R6.F3	UCAACCAUCCAACUCAAGUUGAGAAAUGAACCGCUAGCCC	66
mP40R6.B5	UCAAACAUCYAACUCAAGAYGUUAAAGGARCCGUGAGCS	67
mP40R6.B1	ACGAAGAUCCGAUGGCCCAGAGAAAUCUCCCACUCUGCCC	68
mP40R6.F11	CACCAUAUUCUGUAACGCAGGCUUUCCACAUCGAUCCCCU	69
mP40R6.D9	CCCAAGACACGGGGAAUGGAACUACCACAUCUUAUACAGC CCU	70

What is claimed is

1. A nucleic acid ligand to CD40ligand identified according to a method comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with CD40ligand, wherein nucleic acids having an increased affinity to CD40ligand relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to CD40ligand, whereby a nucleic acid ligand of CD40ligand may be identified.

2. A purified and non-naturally occurring RNA ligand to CD40ligand wherein said ligand is selected from the group consisting of SEQ ID NOS:3-70.

3. A purified and isolated non-naturally occurring nucleic acid ligand to CD40ligand.

4. The nucleic acid ligand of claim 1 wherein said CD40ligand is associated through hydrophobic interactions with a solid support, and wherein steps b)-c) take place on the surface of said solid support.

5. The nucleic acid ligand of claim 4 wherein said solid support is a bead.

6. The nucleic acid ligand of claim 1 wherein said candidate mixture of nucleic acids is comprised of single stranded nucleic acids.

7. The nucleic acid ligand of claim 6 wherein said single stranded nucleic acids are ribonucleic acids.

8. The nucleic acid ligands of claim 6 wherein said single stranded nucleic acids are deoxyribonucleic acids.

9. The nucleic acid ligand of claim 7 wherein said candidate mixture of nucleic acids comprises 2'-F (2'-fluoro) modified ribonucleic acids.

10. The purified and isolated non-naturally occurring nucleic acid ligand of claim 3 wherein said nucleic acid ligand is single stranded.

11. The purified and isolated non-naturally occurring nucleic acid ligand of claim 10 wherein said nucleic acid ligand is RNA.

12. The purified and isolated non-naturally occurring RNA ligand of claim 11 wherein said ligand is comprised of 2'-fluoro (2'-F) modified nucleotides.

13. A method for the isolation of nucleic acid ligands to CD40ligand, comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with CD40ligand, wherein nucleic acids having an increased affinity to CD40ligand relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to CD40ligand, whereby a nucleic acid ligand of CD40ligand may be identified.

14. The method of claim 13 wherein said candidate mixture comprises single-stranded nucleic acids.

15. The method of claim 14 wherein said single-stranded nucleic acids comprise ribonucleic acids.

16. A method for the treatment of a disease resulting from T cell activation, the method comprising administering a nucleic acid ligand to CD40ligand.

17. A method for treating atherosclerosis comprising administering a nucleic acid ligand to CD40ligand.

18. A method for preventing organ, tissue, or graft rejection comprising administering a nucleic acid ligand to CD40 ligand.

19. A pharmaceutical composition for the treatment of atherosclerosis comprising a nucleic acid ligand to CD40ligand and a pharmaceutically acceptable excipient.

20. A pharmaceutical composition for the prevention of organ, tissue or graft rejection comprising a nucleic acid ligand to CD40ligand and a pharmaceutically acceptable excipient.

21. A method for detecting a blood clot in an individual, the method comprising:

(a) providing a nucleic acid ligand to human CD40ligand, said nucleic acid ligand conjugated to a radioactive label;

(b) administering said nucleic acid ligand to said individual; and

(c) detecting the site of said blood clot by analyzing the localization of said nucleic acid ligand using a radioimaging technique.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(72) Inventors; and

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(74) Agents: **SWANSON, Barry, J.** et al.; Swanson & Bratschun, L.L.C., Suite 330, 1745 Shea Center Drive, Highlands Ranch, CO 80129 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

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WO 01/09160 A1

(54) Title: NUCLEIC ACID LIGANDS TO CD40LIGAND

(57) Abstract: Methods are provided for generating nucleic acid ligands of CD40ligand. The methods of the invention use the SELEX method for the isolation of nucleic acid ligands. The invention also includes nucleic acid ligands to CD40ligand, and methods and compositions for the treatment and diagnosis of disease using the nucleic acid ligands.

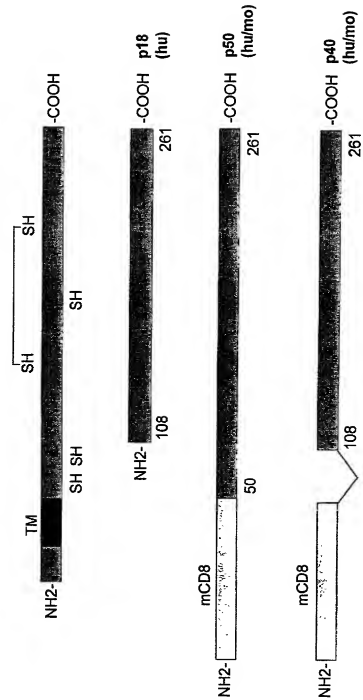
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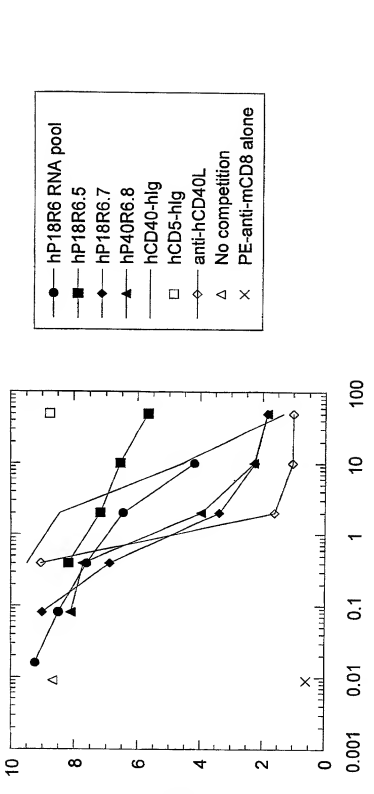
Fig. 1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hCD5-hIg
anti-hCD40L
No competition
PE-anti-mCD8 alone
MCF
0
2
4
6
8
10
0.001
0.01
0.1
1
10
100
[competitor] (ug/ml)

Fig. 2

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hP40R6.1-biotin
hP40R6.4-biotin
hP40R6.8-biotin
hP40R6.40-biotin
hP40R6.48-biotin
anti-hCD40L-biotin
SA-PE alone
MCF
25
20
15
10
5
0
0.0001
0.001
0.01
0.1
1
ug/ml competitor

Fig. 3

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

Docket No. NEX85/PCT-US

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name.

I believe I am the sole, original and first inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Nucleic Acid Ligands to CD40Ligand**, the specification of which:

- ☐ is attached hereto.
- ■ was filed on **July 28, 2000** as United States Application Serial No. _____ or **PCT International Application Number PCT/US00/20625** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.
I hereby claim foreign priority benefits under 35 USC §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.
Prior Foreign Application(s)

Number:	Country:	Date Filed: (day/mo/yr)	Priority not claimed ☐

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below.

Application Number:	Filing Date: (mo/day/yr)

I hereby claim the benefit under 35 USC §120 of any United States application(s), or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 USC §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Number:	Filing Date: (mo/day/yr)	Status - patented, pending, abandoned
09/364,380	29 July 1999	Pending

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Barry J. Swanson, #33,215	Rosemary Kellogg, #39,726	James L. Brown, #48,576
Thomas D. Bratschun, #32,966	Darla G. Yoerg, #48,053	Steven N. Hird, #P-51,112
Margaret M. Wall, #33,462	Dan Shifrin #34,473	Diane Cruz, #33,960
Max D. Hensley, #27,043	Mark Bosse, #35,071	

CUSTOMER NO: 25,871

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Address all correspondence to: Barry J. Swanson
Swanson & Bratschun, L.L.C.
1745 Shea Center Drive, Suite 330
Highlands Ranch, Colorado 80129

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): **ALAN KORMAN**

Inventor's signature: _____ Date: 2/9/2002
Residence: Piedmont, California Citizenship: US
Post Office Address: 301 El Cerrito Avenue
Piedmont, California 94611-4103
CA

■ Additional inventors are being named on separately numbered sheets attached hereto.

Full name of second joint inventor (given name, family name): **LARRY GOLD**

Inventor's signature:_____ Date:_____

Residence: Boulder, Colorado Citizenship: US

Post Office Address: 1033 5th Street

Boulder, Colorado 80302

CO

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Docket No. NEX85/PCT-US

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- ☐ is attached hereto.
- ■ was filed on **July 28, 2000** as United States Application Serial No. _____ or **PCT International Application Number PCT/US00/20625** and was amended on _____ (if applicable).

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Full name of sole or first inventor (given name, family name): **ALAN KORMAN**

Inventor's signature:_____ Date:_____

Residence:	Piedmont, California	Citizenship: US
Post Office Address:	301 El Cerrito Avenue Piedmont, California 94611-4103	

■ Additional inventors are being named on separately numbered sheets attached hereto.

Full name of second joint inventor (given name, family name): LARRY GOLD

Inventor's signature: _____ Date: 1/24/02

Residence: Boulder, Colorado Citizenship: US

Post Office Address: 1033 5th Street
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<223> All pyrimidines are 2'F

<400> 29
gggaggacga ugcggaaccc gggcagucca aucuuucagu cccacaucug cuccccagac 60
gacucgcccg 70

<210> 30
<211> 70
<212> RNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F
N = A, G, 2'-F-U or 2'-F-C

<400> 30
gggaggacga ugcggcaaac cuccacaacc ugcgcaagcu ucccaucnuu cugcccagac 60
gacucgcccg 70

<210> 31
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F

<400> 31
gggaggacga ugcggacguc ucgguugagc ccuuacaauc auuucuguau gcccucagac 60
gacucgcccg 70

<210> 32
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F

<400> 32
gggaggacga ugcggacgaa gacccgaugg cccagagaaa ucucccacuc ugccccagac 60
gacucgcccg 70

<210> 33
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)

<223> All pyrimidines are 2'F

<400> 33
gggaggacga ugcggaagcc cuaagagaau agcccuugac gccuacuccc cugcccagac 60
gacucgcccg 70

<210> 34
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 34
gggaggacga ugcggacaag ugaucugagg aaggaagaca agacucucaa ccacccagac 60
gacucgcccg 70

<210> 35
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> misc_structure
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 35
gggaggacga ugcggacaag agaucugugg aaggaagaca agacucucaa ccacccagac 60
gacucgcccg 70

<210> 36
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 36
gggaggacga ugcggaacuc ucgagaaagg aacaucaaag ugucaaccau ccgucagacg 60
acucgcccg 69

<210> 37
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 37
gggaggacga ugcggcaaga aaggaacguu cagucaacca ucugcuaccg cccucagacg 60
acucgcccg 69

<210> 38
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 38
gggaggacga ugcggcaacu cucgagaaag gaacaucaaa gugucaacca uccgucagac 60
gacucgcccg 70

<210> 39
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 39
gggaggacga ugcgggaacu cucgagaaag gaacaucaaa gugucaacca uccgucagac 60
gacucgcccg 70

<210> 40
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)

<223> All pyrimidine are 2'F

<400> 40
gggaggacga ugcggagaaa ggagagaaaa uccaaccauc ccgaacacgc ucccucagac 60
gacucgcccg 70

<210> 41
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 41
gggaggacga ugcggaggaa ggaacgauag aagucaacca ccccacaguu gcccucagac 60
gacucgcccg 70

<210> 42
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 42
gggaggacga ugcggcaacu cucgagaaag gaacaucaaa gcgucaacca uccgucagac 60
gacucgcccg 70

<210> 43
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 43
gggaggacga ugcggcaacu cucgagaaag gaaucacaua gaucaaccac ccggucagac 60
gacucgcccg 70

<210> 44

<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 44
gggaggacga ugcggcaacu cucgagaaag gaacaucaaa gugucaacca ucugucagac 60
gacucgcccg 70

<210> 45
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 45
gggaggacga ugcggcacug cucgaggaag gaaucacaua gaucaaccac ccgucagacg 60
acucgcccg 69

<210> 46
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 46
gggaggacga ugcggcaaga aaggaacucu aauggucaac caucugcgcg caccucagac 60
gacucgcccg 70

<210> 47
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 47
gggaggacga ugcggcaaga aaggaacguu cagucaacca ucugcuaucg cccucagacg 60
acucgcccg 69

<210> 48
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 48
gggaggacga ugcggcaaga aaggaacguu cagucaacca ucugcuaccg uccucagacg 60
acucgcccg 69

<210> 49
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F

<400> 49
gggaggacga ugcggcaaga aaggaauagc augaccggcu uucaaccauc uggucagacg 60
acucgcccg 69

<210> 50
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 50

gggaggacga ugcggccaag aaaggaauag caugaccggc uuucaaccau cuggucagac 60
gacucgcccg 70

<210> 51
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 51
gggaggacga ugcggccaag aaaggaauag caugacaggc uuucaaccau cuggucagac 60
gacucgcccg 70

<210> 52
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 52
gggaggacga ugcggcucga gaaaggaaca aaggucaacc auccgagccc uaccucagac 60
gacucgcccg 70

<210> 53
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 53
gggaggacga ugcgggcuca gcaagaacaa aggcuggggu aaauuuccga cccgucagac 60
gacucgcccg 70

<210> 54
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 54
gggaggacga ugcgggcuca guaagaacaa aggcuggggu aaauuuccga cccgucagac 60
gacucgcccg 70

<210> 55
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 55
gggaggacga ugcgggcuca gcaagaacaa aggcuggggu aaauuuccaa cccgucagac 60
gacucgcccg 70

<210> 56
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 56
gggaggacga ugcggggaca ccggauaccc cccggcgugu aaauuucaag ccccccagac 60
gacucgcccg 70

<210> 57
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base

<222> (1)..(70)
<223> All pyrimidine are 2'F; M = A or 2'-F-C

<400> 57
gggaggacga ugcggggaca ccggauaccc cccggmgugu aaauumcaag ccccccagac 60
gacucgcccg 70

<210> 58
<211> 68
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(68)
<223> All pyrimidine are 2'F

<400> 58
gggaggacga ugcgggcucc cagagauugc ugggggguaa auuuccaacc cgucagacga 60
cucgcccg 68

<210> 59
<211> 68
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(68)
<223> All pyrimidine are 2'F

<400> 59
gggaggacga ugcgggcucc cagagauugc ugggggguaa auuuccgacc cgucagacga 60
cucgcccg 68

<210> 60
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 60
gggaggacga ugcggucaac cauccaacuc aaguugagaa aggaaccgcu agccccagac 60
gacucgcccg 70

<210> 61
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 61
gggaggacga ugcggucaac cauccaacuc aaguugagaa aggaaccacu agccccagac 60
gacucgcccg 70

<210> 62
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 62
gggaggacga ugcggucaac cauccaacuc aaguugagaa aggaaccacu cgccccagac 60
gacucgcccg 70

<210> 63
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F; N = A, G, 2'-F-U or 2'-F-C

<400> 63
gggaggacga ugcggncann canccancnn aagnngagaa aggaaccacn acccccagac 60
gacucgcccg 70

<210> 64
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 64
gggaggacga ugcggucaac cauccaacuc aaguugagaa aggaaccgcu acccccagac 60
gacucgcccg 70

<210> 65
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 65
gggaggacga ugcggucaac cauccaacuc aaguagagaa aggaaccgcu agccucagac 60
gacucgcccg 70

<210> 66
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 66
gggaggacga ugcggucaac cauccaacuc aaguugagaa augaaccgcu agccccagac 60
gacucgcccg 70

<210> 67
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidine are 2'F; ; Y = 2'-F-U or 2'-F-C; R
= A or G; M; S = G or 2'-F-C

<400> 67
gggaggacga ugcggucaaa caucyaacuc aagayguuaa aggarccgug agcscagacg 60
acucgcccg 69

<210> 68
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 68
gggaggacga ugcggacgaa gauccgaugg cccagagaaa ucucccacuc ugccccagac 60
gacucgcccg 70

<210> 69
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidine are 2'F

<400> 69
gggaggacga ugcggcacca uauucuguaa cgcaggcuuu ccacaucgau ccccucagac 60
gacucgcccg 70

<210> 70
<211> 73
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> modified_base
<222> (1)..(73)
<223> All pyrimidine are 2'F

<400> 70
gggaggacga ugcggcccaa gacacgggga auggaacuac cacaucuuau acagcccuca 60
gacgacucgc ccg 73

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